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TRANSLATION

D E S C R I P T I O N

NUCLEOTIDE SEQUENCES OF CORYNEFORM BACTERIA CODED FOR PROTEINS PARTICIPATING IN L-SERINE METABOLISM AND METHOD FOR MICROBIAL PRODUCTION OF L-SERINE

The invention relates to nucleotide sequences of coryneform bacteria coded for proteins which participate in L-serine metabolism with reduced or omitted L-serine dehydratase and microorganisms for and method of making L-serine.

The amino acid L-serine has been found to be useful in the food industry, the animal feed industry and the pharmaceutical industry as well as in human medicine. It serves as a building block for the synthesis of other industrially valuable products like for example L-tryptophan from indole and L-serine.

It is known that L-serine can be produced by the fermentation of coryneform bacteria strains. Thus for example a strain of *Corynebacterium glycinophilum* is capable of forming L-serine from glycine and carbohydrates (Kubota K, Kageyama K, Shiro T and Okumura S (1971) Journal of General Applications in

Microbiology, 17: 167-168; Kubota K, Kageyama K, Maeyashiki I, Yamada K and Okumura S (1972) Journal of General Applications in Microbiology 18: 365). The enzyme L-serine-hydroxymethyltransferase here participates in the conversion of glycine to L-serine. (Kubota K and Yokozeki K (1989) Journal of Fermentation and Bioengineering, 67(6):387-390). These *Corynebacterium glycinophilum* strains have a defective serine dehydrataze which produces undirected mutagenesis (Kubota K (1985) Improved production of L-serin by mutants of Corynebacterium glycinophilum with less serine dehydratase activity. Agricultural Biological Chemistry, 49:7-12). This enzymatic activity is Pyridoxal 5'-Phosphate dependent and not molecularly characterized. (Kubota K., Yokozeki K, Ozaki H. (1989) Effects of L-serine dehydratase activity on L-serine production by Corynebacterium glycinophilum of an examination of the properties of the enzyme. Agric. Biol. Chem 49:7-12). From US patent 4,528,273 a method of producing L-serine from glycine is known in which the microorganism serine dehydratase is negative.

Furthermore, L-serine can be produced fermentatively from methanol and glycine with the aid of methylotrophic bacteria like for example *Hyphomicrobium* strains (Izumi Y, Yoshida T, Miyazaki SS, Mitsunaga T, Ohshiro T, Shiamo M, Miyata A and Tanabe T (1993) Applied Microbiology and Biotechnology, 39: 427-432). In both cases the amino acid glycine must be introduced as a precursor for the formation of the amino acid L-serine.

In addition, coryneform bacteria are known which can produce the L-serine directly from carbohydrates without further addition of precursors.

This is advantageous for industrial scale economical production of L-serine since the L-serine can be made directly from carbohydrates without the expensive addition of precursors, these strains which belong to the family *Corynebacterium glutamicum* have resistance to the L-serine analog serine hydroxamate and β-chloroalanine and are obtained by undirected mutagenesis (Yoshida H and Nakamaya K (197) NIHON-Nogli-Kagakukaishi 48: 201-208).

There are also *Brevibacterium flavum* strains known which have because of undirected mutagenesis defects in the breakdown of L-serine, an enhanced activity of the *serA* coded 3-phosphoglycerate dehydrogenase and an overexpression of *serB* and *serC* genes deriving from *Escherichia coli* (EP0931833A2).

It is the object of the invention to make available features which will permit improved production of L-serine or metabolic products which derive therefrom like for example tryptophan. It is thus also an object of the invention to provide nucleic acids which code for proteins participating in L-serine metabolism and which by comparison with the proteins derived from the wild type organism show a no decomposition of L-serine to pyruvate or a reduced decomposition of L-serine to pyruvate. In this connection it is a further object of the invention to provide

an L-serine dehydratase as well as microorganisms with an L-serine dehydratase shown to reduce decomposition of L-serine. Further it is an object of the invention to provide an improved method for the microbial production of L-serine.

Starting from the preamble of claim 1, the objects are achieved, in accordance with the invention with the features given in the characterizing clause of claim 1. Furthermore, the objects are achieved starting from the preamble of claim 7 with the features given in the characterizing part of claim 7. The objects are also attained starting from the preamble of claim 8 according to the invention with the features given in the characterizing part of claim 8. The objects are also achieved starting with the preamble of claim 9 with the features given in the characterizing part of claim 9. The objects are also achieved starting with the preamble of claim 14, in accordance with the invention, with the features given in the characterizing part of claim 14. Starting with the preamble of claim 20, the objects are also achieved according to the invention by the features given in the characterizing part of claim 20. Furthermore, the objects are attained according to the invention starting from the preamble of claim 21 by the features of the characterizing part of claim 21.

With the nucleic acids and polypeptides according to the invention it is possible to produce an L-serine dehydratase such that there is a reduced decomposition of L-serine or no longer any

decomposition of L-serine. Furthermore, it is possible to provide microorganisms and a method by which L-serine production can be obtained with higher yield by comparison with hitherto known microbial methods.

Further advantages have been given in the dependent claims.

According to the invention, in microorganisms of the corynebacterium family, replicatable and optionally recombinant nucleic acid is provided with nucleotide sequence coding for the L-serine dehydratase, hereinafter referred to also as SDA, which is partially or completely deleted or mutated or is expressed to a reduced extent by comparison with the naturally occurring nucleotide sequence or is not expressed at all.

The subject of the invention is, further, the provision of nucleic acids whose *sdaA* gene sequence is partially or completely deleted or mutated or has, relative to the naturally available nucleotide sequence reduced expression or which does not express at all. For example the nucleic acids with a nucleotide sequence according to SEQ ID No 1 can have its nucleotides from position 506 to position 918, partly or completely deleted or mutated or can be allele, homologue or derivative of this nucleotide sequence or a nucleotide sequence which hybridizes therewith have been found to be advantageous. In addition, it has

been found to be advantageous for the deletion or mutation of the cystein-containing sequence required for forming the iron-sulfur clusters (Hofmeister et al., (1994) Iron-sulfur cluster-containing L-serine dehydratase from Peptostreptococcus asaccharolyticus: correlation of the cluster type with enzymatic activity. FEBS Letters 351: 416-418) has been found to be advantageous.

The wild type L-serine-dehydratase (*sdaA*) gene sequence is generally known and can be obtained by the artisan from the known data bank (NCBI Accession Nr. AP005279) or from the attached sequence protocol according to SEQ ID No. 1.

The complete deletion of the L-serine dehydratase (*sdaA*) gene can be achieved for example by directed recombinant DNA techniques. Suitable methods for this purpose are found in Schafer et al. (Gene (1994) 145: 69-73) or also Link et al. (Journal of Bacteriology (1998) 179: 6228-6237). Furthermore, only a part of the gene can be deleted or also mutated fragments of the L-serine dehydratase gene can be formed by replacement. By deletion or replacement it is possible to achieve a loss or a reduction in the L-serine dehydratase activity. An example of such a mutant is the *C. glutamicum* strain ATCC133032ΔsdaA which has a deletion in the *sdaA* gene.

To limit the expression of the *sdaA* gene or achieve reduced expression, for example, the promoter and regulatory

regions which are located upstream of the structural gene can be mutated. In a similar manner, expression regulatory cassettes can be built onto the structural gene, upstream thereof. By regulatable promoters it is additionally possible to reduce the expression in the course of fermentative L-serine formation. It is also possible to provide a regulation of the translation in which for the example of stability of the m-RNA is reduced. Furthermore, genes can be used which code for the corresponding enzyme with reduced activity. Alternatively, furthermore, a reduced expression of the L-serine dehydratase gene can be achieved by varying the medium composition and culture conditions. Guides thereto for the artisan can be found among others in Martin et al. (Bio/Technology 5, 137-146 (1987)), by Guerrero et al. (Gene 138, 35-41 (1994)), Tsuchiya and Morinaga (Bio/Technology 6, 428-430 (1988)), Eikmanns et al. (Gene 102, 93-98 (1991)), in the European Patent EPS 0 472 869, US Patent 4,601,893, Schwarzer and Puhler (Bio/Technology 9, 84-87 (1991), Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994), LaBarre et al. (Journal of Bacteriology 175, 1001-1007 (1993)) and in patent application WO 96/15246.

The nucleic acids according to the invention are characterized that they can be isolated from the coryneform bacteria, preferably of corynebacterium or brevibacterium family and especially preferably from *Corynebacterium glutamicum*.

Examples of the coryneform bacteria wild types, from this parental line are for example,

Corynebacterium acetoacidophilum ATCC 13870;

Corynebacterium acetoglutamicum ATCC 15806;

Corynebacterium callunae ATCC 15991;

Corynebacterium glutamicum ATCC 13032;

Brevibacterium divaricatum ATCC 14020;

Brevibacteriium lactofermentum ATCC 13869;

Corynebacterium lilium ATCC 15990;

Brevibacteriiumm flavum ATCC 14067;

Corynebacterium melassecola ATCC 17965;

Brevibacterium saccharolyticum ATCC 14066;

Brevibacterium immariophilum ATCC 14068;

Brevibacterium roseum ATCC 13825;

Brevibacterium thiogenitalis ATCC 19240;

Microbacterium ammoniaphilum ATCC 15354.

Examples of the production of mutants or production strains suitable for the production of L-serine are organisms from the group of Arthrobacter, Pseudomonas, Nocardia, Methylobacterium, Hyphomycrobium, Alcaligenes or Klebsiella. The present invention is characterized more particularly by the naming of the aformentioned bacterial strains but should not be considered limited thereto.

By a "nucleic acid" or a "nucleic acid fragment" there is meant, in accordance with the invention, a polymer of RNA or DNA

which can be single stranded or double stranded and can have optional natural, chemically synthesized, modified or artificial nucleotides. The term "DNA polymer" includes in this case also genomic DNA, cDNA or mixtures thereof.

Under "alleles" are to be understood functional equivalents in accordance with the invention, that is substantially similarly effective nucleotide sequences. Functionally equivalent sequences are such sequences which, in spite of different nucleotide sequences, for example because of the degeneration of the genetic code, still retain the desired function. Functional equivalents thus encompass naturally occurring variants of the sequences described therein as well as synthetic nucleotide sequences, for example those obtained by chemical synthesis and optionally nucleotide sequences matched to the codon requirements of the host organism.

Under a functional equivalent is to be understood especially also natural or synthetic mutations of the original isolated sequence which retain the desired function. Mutations include substitutions, additions, deletions, replacements or insertions of one or more nucleotide residues. Included here are also sense mutations which in the protein plane can result for example from the replacement of conserved amino acids which however do not lead to any basic alteration in the activity of the protein and thus can be considered functionally neutral. This includes modifications of the nucleotide sequence which involve in the

protein plane the N-terminus of a protein without however affecting significantly the function of these proteins.

With the present invention, such nucleotide sequences are encompassed which, by modification of the nucleotide sequences can result in corresponding derivatives. The target of such modification can, for example, be a restriction of the coding sequence contained therein or for example also the insertion of further restriction enzyme cutting sites.

In addition, the present invention includes artificial DNA sequences as long as they, as described above, afford the desired characteristics. Such artificial DNA sequences can, for example, be those obtained by reverse translation from proteins established by means of computer supported programming (molecular modeling) or by in vitro selection. Especially suitable are coded DNA sequences which, by reverse translation, can produce a polypeptide sequence which has a specific code on utilization for the host organism. The specific code on utilization can be easily determined by molecular genetic methods common in the art using computer evaluations from other previously known genes of the organism to be transformed.

"Homologous sequences" are to be understood in accordance with the invention to be those sequences which are complementary to the nucleotide sequences according to the invention and/or such sequences which can hybridize with them. The hybridizing sequences include, according to the invention, substantially similar

nucleotide sequences from the group of DNA or RNA which under stringent conditions known per se undergo a specific interaction (binding) of the aforementioned nucleotide sequences. In this category are to be counted also short nucleotide sequences with a length of for example 10 to 30 and preferably 12 to 15 nucleotides. These include according to the invention among others, also so-called primers or probes.

Included in the invention are also the coding regions (structure genes) and preceding (5' or upstream) sequence regions and/or following (3' or downstream) sequence regions. Especially in this category are sequence regions with regulatory functions. They can influence the transcription, the RNA stability or RNA processing as well as the translation. Example of regulatory sequences are, among others, promoters, enhancers, operators, terminators or translation amplifiers.

The subject of the invention is in addition a gene structure containing at least one of the aforedescribed nucleotide sequences and regulatory sequences operatively linked therewith which control expression of the coded sequences in the host cell.

In addition the present invention relates to a vector containing a nucleotide sequence of the aforedescribed kind with its regulator nucleotide sequence operatively linked thereto as well as additional nucleotide sequences for the selection of host cells capable of effecting transformation, for replication within the host cell or for integration in the corresponding host cell genome. In addition, the vector according to the invention can

contain a genome structure of the aforedescribed type. Suitable vectors are those which replicate in coryneform bacteria like for example pZ1 (Menkel E, Thierbach G, Eggeling L, Sahm H., 1989, *Appl Environ Microbiol* 55(3): 684-688), pEKEx2 (Eikmanns et al., Gene 102: 93-98 (1991), or pXMJ19 (Jacoby M., Burkovski A (1999) Construction and application of new *Corynebacterium glutamicum* vectors, Biotechnol. Technique 13:437-441). Other plasmid vectors can be used in the same manner. These identifications are however not limiting for the present invention.

Utilizing the nucleic acid sequence according to the invention, corresponding probes or primers can be synthesized and used, for example, to amplify and isolate analogous genes from other microorganisms, preferably coryneform bacteria, for example with the aid of the PCR technique.

The subject matter of the present invention is thus also a probe for identifying and/or isolating genes coded for proteins participating in the biosynthesis of L-serine, whereby these probes are produced starting from the nucleic acid sequences according to the invention of the aforedescribed type and which contain a suitable marker for detection. In the probe, a partial segment of the sequences according to the invention, for example a conserved region, can be used which for example has a length of 10 to 30 or preferably 12 to 15 nucleotides and under stringent conditions can hybridize with homologous nitride sequences. Numerous suitable markers are known from the literature. The skilled worker in he art can be guided thereto by among others the Handbook of Gait:

Oligonucleotide synthesis: a practical approach (IRL Press, Oxford, UK, 1984) and Newton and Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Deutschland, 1994) or for example, the Handbook "The DIG System Users Guide for Filter Hybridization" the Firma Roche Diagnostics (Mannheim, Deutschland) and Liebl et al. (International Journal of Systematic Bacteriology (1991) 41: 255:260).

The subject matter of the present invention includes, further, an L-serine dehydratase which shows reduced L-serine decomposition by comparison with the wild type L-serine dehydratase and which is coded by a nucleic acid sequence according to the invention or its variants of the aforedescribed type. The present invention thus includes an L-serine dehydratase or an L-serine dehydratase mutant with an amino acid sequence in accordance with sequence ID No. 2 whose amino acids from position 135 to position 274, for example, as a consequence of a directed mutagenesis in the DNA plane, is altered or is a modified form of this polypeptide sequence or an isoform thereof or a mixture thereof. By "altered" in the framework of the present invention one should understand that complete or partial removal or replacement of the amino acids from position 135 to position 274 is contemplated.

Under isoforms we understand enzymes with the same or comparable substrate specificity and effectiveness specificity but which differ with respect to the primary structure.

Under modified forms are to be understood enzymes according to the invention with changes in the sequence, for example, at the N-terminus or C-terminus of the polypeptide or in

the regions of the conserved amino acids without however negatively affecting the function of the enzyme. These changes can be in the form of amino acid replacement in accordance with methods known per se.

The polypeptides according to the invention are characterized by the fact that they derive from coryneform bacteria and preferably are of the corynebacterium or brevibacterium family and especially of the *Corynebacterium glutamicum* or Brevibacterium types and especially preferably derive from *Corynebacterium glutamicum*. Examples of the coryneform bacteria in the strain culture of the wild type are for instance

Corynebacterium acetoacidophilum ATCC 13870;
Corynebacterium acetoglutamicum ATCC 15806;
Corynebacterium callunae ATCC 15991;
Corynebacterium glutamicum AT CC 13032;
Brevibacterium divaricatum ATCC 14020;
Brevibacterium lactofermentum ATCC 13869;
Corynebacterium lilium ATCC 15990;
Brevibacterium flavum ATCC 14067;
Corynebacterium melassecola ATCC 17965;
Brevibacterium saccharolyticum ATCC 14066;
Brevibacteriium immariophilum ATCC 14068;
Brevibacterium roseum ATCC 13825;
Brevibacterium thiogenitalis ATCC 19240; and
Microbacterium ammoniaphilum ATCC 15354.

Examples of mutants or production strands suitable for the production of L-serine are organisms from the group of arthrobacter, pseudomonas, nocardia, methylobacterium, hyphomycrobium, alcaligenes or klebsiella. The present invention has been characterized by listing the aformentioned bacteria strains, but this list should not be considered limiting of the invention.

The present invention comprises, further, a genetically altered microorganism characterized in that it contains a nucleotide sequence coding for the L-serine dehydratase which is in part or completely deleted or mutated or expressed to a reduced extent by comparison with the naturally occurring nucleotide sequence or which is not expressed at all.

The invention comprises further a microorganism which is characterized in that the *sdaA* gene is partially or completely deleted or mutated or which is expressed to a reduced extent by comparison with the naturally occurring *sdaA* gene or which is not expressed at all.

The invention encompasses as well a genetically altered microorganism containing in replicatable form a gene structure or a vector of the aforedescribed type.

The subject of the present invention is moreover also a genetically modified microorganism containing a polypepetide according to the invention of the aforedescribed type and which in

comparison to the corresponding genetically unmodified microorganism has reduced or no L-serine decomposition.

A microorganism which, according to the invention has been genetically modified is characterized further in that it is a coryneform bacterium, preferably of the family Corynebacterium or Brevibacterium and especially preferably of the species *Corynebacterium glutamicum* or *Brevibacterium flavum*.

Basically the genes can, using methods known per se like for example the polymerase chain reaction (PCR), be amplified by the aid of short synthetic nucleotide sequences (primers) and then isolated. The production of the primers used can be effected generally based upon known gene sequences from existing homologies in conserved regions of the gene and/or taking into consideration the GC content of the DNA of the microorganism investigated.

A further procedure for isolating coding nucleotide sequences is the complementation of so-called defect mutants of the organism to be investigated which at least phenotypically show a function drop in the activity of the gene investigated or the corresponding protein. Under a complementation is to be understood the preservation of the gene defect of the mutant and the substantial reproduction of the original configuration before mutagenesis which can be achieved by the insertion of functional genes or gene fragments from the microorganism to be investigated.

A classical mutagenesis process for producing defect mutants or mutants with a reduced L-serine dehydratase or an L-serine dehydratase which has been shut down is for example the treatment of the bacteria cell with chemicals like for example N-Methyl-N-Nitro-N-Nitrosoguanidine or the use of UV radiation. Such methods of mutation resolution are generally known and can be found among others in Miller (A Short Course in Bacterial Genetics, A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria (Cold Spring Harbor Laboratory Press, 1992)) or the Handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981)).

The present invention relates moreover to a method for the microbial production of L-serine whereby the nucleic acids in the microorganisms which code for the L-serine dehydratase in part or completely are deleted or mutated or expressed to a lesser extent or practically not at all by comparison with the naturally available nucleic acids, using these genetically altered microorganisms for the microbial production of L-serine, and isolating the correspondingly formed L-serine from the culture medium.

The genetically altered microorganisms produced in accordance with the invention can be used for the purpose of culturing L-serine in continuous cultures or discontinuously in batch processes (set cultivation) or in a fed batch process or a

repeated fed batch process. A collection of known cultivation methods can be found in the textbook of Chmiel (Bioprozesstechnik 1. Einfuhrung in die Bioverfahrensechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium used must be suitable to suitably satisfy the requirements of the respective strain. Descriptions of culture media for various microorganisms can be found in the handbook "Manual of Methods for General Bacteriology" der American Society for Bacteriology" der American Society for baceriology (Washington D.C., USA, 1981) as carbon sources, sugars and carbohydrates like for example glucose, saccharose, lactose, fructose, maltose, molasses, starch and cellulose can be used, oils and fats like for example soy oil, soy flour oil, peanut oil, cocoanut fats can be used, fatty acids like for example palmitic acid, stearic acid and linolaic acids can be used, alcohols like for example glycerine and ethanol can be used and organic acids like for example acetic acid can be used. These substances can be employed individually or as mixtures. As nitrogen sources, organic nitrogen containing compounds like peptones, yeast extract, meat extract, malt extract, corn spring water, soybean meal and urea, or inorganic compounds like ammonium sulfate, ammonium chloride ammonium phosphate, ammonium carbonate and ammonium nitride are used. The nitrogen sources can be used individually or as mixtures. As phoshorous sources, phosphoric acid, potasiuim

dihydrogen phosphate or dipotassium phosphate or the corresponding sodium-containing salts are used. The culture medium must contain further salts of metal like for example magnesium sulfate or iron sulfate which are required for growth. Finally essential nutrients like amino acids and vitamins are added to the above-mentioned substances. The culture medium can in addition have suitable precursors added to it. The additives can be introduced into the culture in the form of one time addition or can be fed to the culture suitably during cultivation. For pH control of the culture basic compounds like sodium hydroxide, potassium hydroxide, ammonia or acqueous ammonia can be used or acid compounds like phosphoric acid or sulfuric acid can be used in a suitable way. For control of foaming, antifoaming agents like for example fatty acid polyglycol esters can be used. To maintain the stability of plasmids suitable selectively effective substances, for example antibiotics can be added to the medium. To maintain the aerobic conditions, oxygen or oxygen-containing mixtures like for example air are introduced into the culture. The temperature of the culture is normally between 20°C and 45°C and preferably 25°C to 40°C. The culture is maintained for a duration until L-serine production is a maximum. This duration is normally from 10 hours to 160 hours.

The analysis of the L-serine formation can be carried out by anion exchange chromatography with subsequent ninhydrin derivatization as described by Spackman et al. (Analytical

Chemistry, 30 (1958), 1190) or the analysis can be effected by reverse phase HPLC as described by Lindroth et al. (Analytical Chemistry (1979) 51: 1167-1174.

The microorganisms which are the subject of the present invention can produce L-serine from glucose, saccharose, lactose, mannose, fructose, maltose, molasses, starch, cellulose or from glycerine and ethanol. The method can use the coryneforme bacteria representatives which have already been described in detail. A selection of the results of the fermentation has been given in Table 1. The genetically altered microorganisms of the invention show a substantially improved L-serine production by comparison with the corrsponding nontransformed microorganism (wild type) or the micororganisms which contain only the vector without the gene insert. In a special variation of the present invention it has been shown that *C. Glutamicum* ATCC 13032ΔpanBCΔ*sdaA* gives rise to at least a 4-fold increase in the L-serine accomulation in the medium by comparison with the control strain (Table 1). Through the common overexpressionof other genes, which act positively on the L-serine biosynthesis pathway, a 16-fold increase in L-serine production can be achieved.

Amino acid production strains, in accordance with the present invention should be understood to be *Corynebacterium glutamicum* strains or homologous microrganisms which are altered by classical and/or molecular genetic methods so that metabolic flow

is amplified in the direction of the biosynthesis ofamino acids or theirderivatives (metabolic engineering). For example, with these amino acid production strains, one or more genes and/or the corresponding enzyme have their regulation altered or are rendered deregulated at different and correspondingly complex regulated key positions in the metabolic pathway. The present invention includes thereby all such already known amino acid production strains preferably of the corynebacterium family or homologous organisms. Further, such production strains are encompassed within the invention which the skilled worker in the art will recognize by analogy with other microorganisms, for example, enterobacteria, bacillaceen or yeast types can be produced by current methods.

The Figures show examples of plasmids which can be used as well as experimental results with respect to nucleic acids or microorganisms according to the invention.

It shows:

FIG. 1 The integration plasmid pK19mobsacB-DeltasdaA Markings on the outer edge of the plasmid indicate the respective restriction sites. The portion within the circle indicates the following gene:

kan	canamycin resistance
sacB	Sucrase
OriT	Transfer origin

sdA'	5' end of the *sdaA* gene
sda"	3' end of the *sdaA* gene

FIG. 2: A graph of the ratio between growth (square symbol □) and L-serine breakdown (circle symbol ○) of *C. glutamicum 13032ΔpanBCΔsdaA*, clone 1 (□, ○) and *C. glutamicum* 13032ΔpanBCΔsdaA, clone 2 (■, ●) compared with *C. glutamicum* 13032ΔpanBC, clone 1 (□, ○) and *C. glutamicum* 13032ΔpanBC,clone 2 (■, ●). The abscissa X represents the fermentation in hours (h). The ordinate y_1 is the growth of the microorganisms measured in terms of optical density at 600 nm. The ordinate Y_2 gives the L-serine concentration in mM.

FIG 3: The expression plasmid pEC-T18mob2-serA^{fbr}CB. The indicia on the outer edge of the plasmid show the resective restriction sites. The indicia within the circle represent the following genes:

SerC	Phosphoserine Transaminase
SerB	Phsophoserine Phosphatase
Rep	Replication origin
Per	Partition cell partition gene
Tet	Tetracycline resistance gene
RP4-mob	Mobilizaiton origin
OriV	Source of DNA replication
SerA-fbr	3-phosphoglycerate dehydrogenase

Examples:

1. The construction of sdaA-deletion mutant of *C. glutamicum* ATCC13032 ΔpanBC.

The starting point was *Corynebacterium glutamicum* with a nuclotide sequence (Genbank-Accession-Number BAB99038; SEQ-ID-No. 1) whose derivative polypeptide sequence showed 40% identity with the described L-serine dehydratase of *E.coli* (NCBI-Accession-Number P1095). By gene protected mutagenesis by the method of Link et al (Link AJ, Phillips D, Church GM, Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. J. Bacteriol. 1997 Oct;179(20):6228-37) and Schafer et al. (Gene 145: 69-73 (1994)) the *sdaA*-gene of *C. glutamicum* was deleted. The following primers wre derived from the sdaA corynebacterial sequence (NCBI Accession-Number AP005279):

sdaA-1: 5'-TCGTGCAACTTCAGACTC-3'
(AP005279 nucleotide 73635 - 73653);

sdaA-2: 5'-CCCATCCACTAAACTTAAACACGTCATAATGAACCCACC-3'
(AP005279 complementary to nucleotide 74121-74139);

sdaA-3: 5'-TGTTTAAGTTTAGTGGATGGGCCGACTAATGGTGCTGCG-3'
(AP005279 complementary to nucleotide 74553 - 74571);

sdaA-4: 5'-CGGGAAGCCCAAGGTGGT-3'
(AP005279 nucleotide 75044 - 75062)

Primers sdaA-1 and sdaA-2 flank respectively the beginning and the end of the sdaA-3 make available respective complementary linker regions (see relevant text) which enable in a two-stage PCR process (cross over PCR) a deletion of the *sdaA* gene in vitro. In a first PCR reaction with the chromosomal DNA of *C. glutamicum*, the primer combination sdaA-1 and sdaA-2 as well as sdaA-3 and sdaA-4 are used. The PCR reaction is carried out in 30 cycles in the presence of 200 µm deoxynucleotide triphosphates (dATP, dCTkP, dGTP, dTTP), each with 600 nM of the corresponding oligonucleotide sdaA-1 and sdaA-4 as well as 60 nM of oligonucleotide sdaA-2 and sdaA-3, 100 ng of chromosomal DNA from *Corynebacterium glutamicum* ATCC13032, 1/10 volumes 10-fold of reagion buffer and 2.6 units of heat stabilized Taq-/Owi-DNA-Polymerase-Mischung mixture (Expand High Fidelity PCR System of Firm of Roche Diagnostics, Mannheim, Deutschland) in a Sthermocycler (PTC-100, MJ Research, Inc., Watertown, USA) under the following conditions: 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 40 seconds. The elongation step at 42°C was extended after 10 cycles by about 5 seconds per cycle. After the PCR reaction, the DNA fragments containing each having a lengthof 500 bp were isolated with QIAExII Gelextraction kit (Qiagen) in accordance with the requirements of the manufacturer on an 0.8% agarose gel and both fragments were used as templates in the second

PCR. As primers the primers sdaA-1 and sdaA-4 were used. This time the reaction was carried out in 35 cycles in the presence of 200 µm deoxynucleotide triphosphates, 600 nM each of the corresponding olegonitrides, 2- mg each of the isolated template DNA from the first PCR, 1/10 volume of 10 fold reaction buffer and 2.6 units of Taq-/Pwo-DNA-Polymerase mixture under the following conditions: 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 80 seconds. Again the elongation steps after 10 cycles were extended by 5 seconds each. After PCR reaction to 1000 bp long DNA fragments which contain the inactive sdaA gene with a 420 bp long central deletion was isolated on a 0.8% agarose gel and cloned, blunt end with the aid of a Sure Clone Kit (Amersham Pharmacia Biotech) in the *Sma*I-restriction site of the inactivation vector pk19mobsacB (Schafer et al Gene 145: 69-73 (1994) which can replicate only in an *E.coli* but not in *C. glutamicum*. The obtained plasmid pK19mobsacB_ΔsdaA (FIG. 1) is tested by restriction mapping for correctness. The cloning was effected in the *Escherichia coli* strain DH5αmcr (Grant et al., Proceedings of the National Academy of Sciences of the United States of America USA (1990) 87: 4645-4649).

Then the plasmid is incorporated by electroporation in *C. glutamicum* 13032ΔpanBC (Radmacher E, Vaitsikova A, Burger U, Krumbach K, Sahm H, Eggeling L. Linking central metabolism with increased pathway flux: L-valine accumulated by *Corynebacterium glutamicum*. Appl Environ Microbiol. 2002 68(5):2246-50) and

subject to selection with integration of the vector. This strain is pantothenate auxotropic as a result of the deletion of the pantothenate biosynthesis genes *panB* and *panC* and is characterized in that it has an amplified accumulation of pyruvate about 50 mM alanin and 8 mM valine because of the pantothenate limitation. In addition the strain can form about 100 µM L-serine and is suitable as a starting strain for the construction of L-serine producers. It contains Kanamycin resistant clones of *C. Glutamicum* 13032ΔpanBC by which inactivation vector is integrated in the genome. To allow selection of the excision of the vector, kanamycin-resistant clones are plated out on saccharose containing LB medium (Sambrook et al., Molecular cloning. A laboratory manual (1989) Cold Spring Harbour Laboratory (Press) with 15 g/l Agar, 2% glucose/ 10% saccharose) and colonies are obtained in which the vector has again been lost as a result of a second recombination event. (Jager et al. 1992, Journal of Bacteriology 174: 5462-5465). Two of these clones whose mucleotides have sdaA genes deleted from positions 506 to 918 are designated and 13032ΔpanBCΔsdaA, clone 1 and 13032ΔpanBCΔsdaA, clone 2 and are used in the further investigations.

2. The influence of the *sdaA* deletion upon L-serine decomposition

In the following, a test was made whether the deleted sdaA gene indeed participates in L-serine decomposition. For this purpose a growth experiment was carried out with each of the two

clones of the strains *C. glutamicum* 13032ΔpanBCΔsdaA in comparison with strain *C. glutamicum* 13032ΔpanBC on minimal medium (Keilhauser et al., Journal of Bacteriology 175 (1993) 5595-5603) which additionally contains 2% glucose 1 μM pantothenate and 100 mM L-serine. The growth and consumption of L-serine were followed. The results are given in FIG. 2.

The results in FIG. 2 show that the deletion of the sdaA genes results in about 40% reduced decomposition of L-serine.

3. Influence of the deletion of the *sdaA* gene on L-serine formation

To test what the influence was of the deletion of the L-serine dehydratase gene upon L-serine formation the strains 13032ΔpanBCΔsdaA (clone 1, clone 2) and 13032ΔpanBC (clone 1, clone 2) with the plasmid pec-T18mob2-sera^{fbr}serCserB the plasmid is formed (FIG. 3) from the vector pEC-T18mob2 (Tauch, A., Kirchner, O., Loffler, B., Gotker, S., Puhler A., and Kalinowski J. Efficient Electrotransformation of Corynebacterium diphtheria with a MiniReplicon Derived from the Cornyebacterium glutamicum Plasmid pGA1. Curr. Microbiol. 45(5), 362-367 (2002)), of the corynebacterial gene serA^{fbr} (Peters-Wendisch P., Netzer R, Eggeling L. Sahm H. 3-Phosphoglycerate dehydrogenase from Corynebacterium glutamicum: the C-terminal domain is not essential for activity but is required for inhibition by L-serine. Appl Microbiol Biotechnol.

2-2 Dec;60(4);437-41) as well as serC and serB (German ptent application 100 44 831.3 of 11 September 2000.

After electroporation, the strains 13032ΔpanBCΔsdaApSerA^{fbr}CB and 13032ΔpanBCp*SerA*^{fbr}CB were obtained.

For testing L-serine output the two strains 13032ΔpanBCp*SerA*^{fbr}CB are cultivated in complex medium (CgIII with 2% glucose and 5 µg/l tetracycline) and the fermentation medium CGXII (J Bacteriol (1993) 175: 5595-5603), each seeded from the preculture to the medium contained in addition 50 µg/l kanamycin nd 1 µM pantothenate. As controls, the two starting strains 13032ΔpanBC and 13032ΔpanBCΔsdaA were cultured in the same manner although the medium did not contain tetracycline. For each at least two independent fermentations were carried out. After culturing for 30 hours at 30°C of a rotating shaker at 120 RPM, the L-serine quantity accumulated in the medium was determined. The determination of the amino acid concentration was carried out by means of high presssure liquid chromatography (J Chromat (1983) 266: 471-482). The results of the fermentation are shown in Table 1 and indicate that the exclusion of L-serine dehydratase led to a 4-fold increase in the L-serine accumulation in the medium independently of whether the L-serine biosynthesis genes *serA*^{fbr}, *serC* and *serB* were overexpressed. The overexpression of the L-serine biosynthesis genes serA^{fbr,} *serC and serB* however resulted in 16 fold increase in L-serine accumulation in the culture

supernatent generally. Thus the use of the constructed and described deletion mutant ΔsdaA resulted in a method which improved the L-serine formation decisively.

Table 1: Accumulation of L-serine in the culture supernatent of *Corynebacterium glutamicum* 13032ΔpanBC and 13032ΔpanBCΔsdaA after expression of the genes *serA*^{fbr}, *serC* and *serB*

Strain	OD_{600}	L-Serine [mM]
13032ΔpanBC	40	0.1
13032ΔpanBCΔsdaA	42	0.4
13032ΔpanBCpserA^{fbr}CB	30	1.6
13032ΔpanBCΔsdaApserA^{fbr}CB	30	6.6

4. Determination of the L-serine Dehydratase Activity

For determining the L-serine dehydratase activity the wild type strands WT pXMJ19 (Jacoby M., Burkovski A (1999) Construction and application of new *Corynebacterium glutamicum* vectors. Biotechnol. Technique 13:437-441), overexpression strand WT pXMJ19_*sdA*
and the deletion strains ΔsdaA pXMJ19 were cultured in CgXII minimal medium as in Keilhauer et al., (1993). The medium contained 30 mg/l protocatechuic acid, 100 mM glucose and 100 mM L-serine. The cells were cultivated in the presence of 1 mM isopropyl-beta-D-thiogalactopyranoside and in the exponential

growth phse at an optical density of 6-8, measured by a Pharmacia Biotech ultrospec 3000 spectral photometer were harvested. They were then centrifuged for 10 minutes at 4500 rpm and 4°C, suspended in 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer (pH 8.0) and centrifuged again. Thereafter the cells were taken up in 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonicacid buffer (pH 8.0), 1 mM $FeSO_4$ and 10mM dithiothreitol. The cell breakdown was effected by means of ultrasonic treatment (Branson sonifier 250; duty cycle 25%, output control 2.5, 10 minutes) on ice.

To determine the L-serine dehydratase activity the reaction set contained 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer (pH 8.0), 10 mM dithiothreitol and 10-100 µl new extract. The detection of the pyruvate formation from the serine was effected as described (Ohmori et al., 1991). The reaction was started by adding 50 mM L-serine and after 10 minutes was stopped by the addition of 1,2-diamino-4,5-dimethoxybenzene reagent in a ratio of 1:1. The reagent, as described in Ohmori et al 1991 was comprised of 4 mg 1,2-diamino-4,5-dimethoxybenzol dissolved in 42.4 ml H_2O, 3.5 ml β-mercaptoethanol and 4.1 ml HCl (37%ig) then incubation was carried out for 2 hours at 102°dry heat.

Detection and quantification of the 2-hydroxy-6,7-dimethoxy-3-methylquinoxaline derivative produced by the pyruvate was carried out by means of high pressure liquid chromatography

also as described. (Ohmori et al., 1991). The protein determination in the raw extract followed by means of the Bradford method (Bradford 1976) using the protein assays (The firm Bio-Rad). The specific L-serine dehydratase activity of the two strands are given in Table 2.

Table 2: Specific Activity of the L-Serine Dehydratase in the Strains
13032 WT pXMJ19_sdaA (Overexpressed), 13032 WT pXMJ19 (Wild type with empty vectors) and 13032 ΔsdaA pXMJ19.

C. Glutamicum Strain	spec. Activity [nmol/min*mg]
13032 WT pXMJ19_sdaA	0.221
13032 WT pXMJ19	0.003
13032 Δ*sdaA* pXMJ19	0